University of Alberta

Biotic Constraints of Western Gall Rust
(Endocronartium harknessii)

by

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Abstract

Two biotic constraints of western gall rust (*Endocronartium harknessii*) of lodgepole pine (*Pinus contorta* var. *latifolia*), age-related host resistance and the mycoparasite *Scytalidium uredinicola*, were studied. In response to field inoculation, the percentage of infected trees and the number of galls per 10 cm of shoot length decreased by 85 and 88%, respectively, as tree age increased from 2 to 10 years. Because the inoculum levels were controlled and the spore germination was independent of tree age, it was concluded that tree resistance increased with age. The shoots of tall trees had thicker epidermal cell wall than those of short trees, suggesting that the chances of infection in tall, old trees may be largely reduced by the epidermal resistance. *Scytalidium uredinicola* appeared to be compatible with gall tissue except for slightly reducing periderm thickness, suggesting it may act as a gall endophyte to protect lodgepole pine from western gall rust.

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I. Literature review and research objectives

Knowledge of natural constraints of forest pathogens could lead to direct silvicultural methods to reduce disease impact. Such information is also valuable for understanding the influence of preexisting constraints on the efficiency of other disease control measures. Western gall rust (*Endocronartium harknessii* (J.P. Moore) Y. Hiratsuka (= *Peridermium harknessii* J. P. Moore)) is an important disease of hard pines in North America (Hiratsuka and Powell 1976). The life cycle, infection mechanisms, infection-limiting factors such as genetic and developmental resistance, and potential biological control agents have been studied for this disease and a number of control measures have been suggested. To reduce disease impact by intensive management, more information on biotic constaints such as age-related resistance and the mycoparasite effect on the rust gall development is needed.

Hosts and distribution

Western gall rust was discovered by H. W. Harkness in 1876 on ponderosa pine (*Pinus ponderosa* Laws.) in Colfax, California (Peterson 1967). It is native on lodgepole pine (*P. contorta* Dougl. ex Loud. var. *latifolia* Engelm.), ponderosa pine and jack pine (*P. banksiana* Lamb.) throughout the Rocky Mountains. At least 22 native and exotic pine species are reported to be susceptible to western gall rust (Peterson 1967). A gall rust on Scots pine (*P. sylvestris* L.) near Woodgate, New York, was described in

1926 as Woodgate-*Peridermium* (York 1926). This gall rust was later recognized to be identical to western gall rust (Boyce 1957; Krebill 1970). Western gall rust ranges from Yukon southward through the Rocky Mountains to northern Mexico, eastward across Canada and the northern United States to the Atlantic Coast (Peterson 1967; Hiratsuka and Powell 1976; Merrill and Wenner 1985). On jack pine, its northeast range overlaps with the southern range of eastern gall rust (*Cronartium quercuum* (Berk.) Miyabe ex Shirai in the Lake States (Anderson 1965; Anderson 1968). Although the two gall rusts produce similar symptoms, they are quite different in the cytological characteristics of spore germ tubes (Anderson and French 1965a; Hiratsuka and Maruyama 1968). In western Canada, western gall rust is the most common and destructive stem rust of lodgepole pine and jack pine forests (Ziller 1974).

Life cycle, cytology and taxonomy

The autoecious nature of the western gall rust fungus was first demonstrated on Monterey pine (*P. radiata* D. Don.) (Meinecke 1916). There are also indications that the fungus might be facultatively heteroecious, based on inoculations of *Castilleja* species, although others have had no success in inoculation of similar plants (Weir and Hubert 1917; Meinecke 1929; Anderson and French 1965b; Peterson 1967). In Canada, both inoculation tests and cytological studies suggest that the fungus is purely autoecious (Zalasky and Riley 1963; Ziller 1970; Hiratsuka and Powell 1976).

The western gall rust fungus primarily produces only one type of fruiting structures-peridermioid telia, which morphologically resemble the typical aecia of *Cronartium* species but produce spores which germinate to produce metabasidia and re-infect the pine host (Hiratsuka at al.1966; Hopkin at al. 1988). Spermogonia are rarely formed, and their formation appears unnecessary for completion of the life cycle of the fungus (Walla et al. 1991; Crane et al 1995).

The mycelium in the gall tissues, at the base of the aecial primordium and in the surrounding hyphal mats, is monokaryatic. Dikaryotization occurs during aecia development, resulting in the binucleate condition in several cell layers at the bottom of the aecium (Hiratsuka and Powell 1976; Crane et al. 1995). The binucleate condition is maintained in very young spores, whereas mature spores are mostly uninucleate before germination (Hiratsuka et al. 1966; Hiratsuka 1991). Spores germinate to produce typically one germ tube. The germ tube has determinate growth and usually separates into three, four, or five segments with usually one nucleus per cell. A side branch is commonly formed from the first cell of the germ tube. One germ tube may produce as many as three branches, one from each segment (Hiratsuka et al. 1966). The side branches, as well as tips, of the germ tubes are capable of causing infection (Hopkin at al. 1988; Hiratsuka 1991). In recognition of the endocyclic life cycle, a new genus, *Endocronartium*, was proposed to accommodate endocyclic species having morphological similarities to *Peridermium* species (Hiratsuka et al. 1966; Hiratsuka 1969), although the

taxonomic treatment of this group of fungi is still in dispute (Hiratsuka 1991, 1998).

Infection process

It has been well demonstrated that infection can occur on primary pine shoot tissue without wounding (True 1938; Quick 1966; Parmeter and Newhook 1967; Allen and Hiratsuka 1985). Direct infection through epidermis occurs on the young shoots of Scots pine and on the hypocotyl of lodgepole and jack pine seedlings (True 1938; Hopkin at al. 1988). In the case of cells penetrated by germ tubes, the thickness of the outer walls was at times much less than for un-penetrated neighbouring epidermal cells. Penetration of epidermis is either intercellular or, more frequently, intracellular through the less lignified portion of the cell walls (True 1938). On seedlings of lodgepole and jack pine, penetration of epidermal cells by germ tubes of *E. harknessii* occurred near the junction of the epidermal cells but not immediately at the junction where the cuticle was thickest (Hopkin at al. 1988). These observations suggest that thin cuticle facilitates germ tube penetration through the shoot surface. Such susceptible areas of tissue may occur before periderm formation along the length of the exposed shoot surface, except at the bases of needle fascicles where the walls of the epidermal cells, and even the outer walls of subepidermal cells, become lignified (True 1938; Moltzan et al. 2001a).

Once the infecting hypha passes from the epidermal cell into the intercellular space below, it branches readily and sends hyphae into neighboring epidermal and subepidermal cells. These hyphae appear to function as haustoria, but such intracellular development is short-lived and the mycelium soon establishes itself intercellularly with typical haustoria (True 1938). In the cortex, the hyphae advance in different directions. Some run parallel to the axis of the twig in the spaces between the first one or two cortical cell layers or just beneath the epidermis. The major portion of the mycelium, however, spreads radially inward through the outer cortex until the inner cortex is reached and there a second vertical spread occurs, which is most pronounced in the intercellular spaces adjacent to and near the epithelial cells of the resin ducts. The mycelium often spreads a considerable distance around the margin of the phloem before entering it. The mycelium moves through the phloem mostly along the phloem rays, crosses the cambium at right angles and continues its radial path mostly along the medullary rays for a short distance into the xylem. The cambium usually is first penetrated at a point or over a limited area adjacent to the point of infection, and subsequent cambium invasion comes always from the phloem, never from vertical or peripheral spread of the mycelium within the cambium itself (True 1938).

Disease development and impact

Disease development

Western gall rust ultimately induces conspicuous globose woody galls on the infected stems of its host. Before a conspicuous swelling appears, there are virtually no consistent early recognizable symptoms that indicate the successful establishment of the fungus. In Scots pine, the infection points are often marked by spots that usually range from orange to dark brown. Their margins are often sharply defined, but frequently are less definite, and may appear water-soaked. Infections whose spots show indefinite or water-soaked margins seem more apt to produce galls than those with more sharply delimited spots (True 1938). In lodgepole pine, the infection spots appear at the early stage of infection as red stain or early pigmentation. In many cases, there are no such early visible symptoms; or if they occur, most of them usually fade away with time. Two subsequent symptoms, red fleck and red streak, which are often superimposed on the red stain symptoms, occur after the early stage of infection (Allen et al. 1990; Kojwang and van der Kamp 1992). Although a high frequency of the general red stain and red flecks symptoms is associated with susceptible families, the occurrence of early symptoms on individual seedlings does not necessarily indicate that gall formation will occur (Kojwang and van der Kamp 1992).

Conspicuous gall formation usually does not appear on the infected shoots until near the end of the following growing season (Merrill and Wenner 1988). The rust galls start to sporulate after they are 2 to 3 years old. The rust sporulation occurs in spring. Rust aecia develop beneath the periderm, which then ruptures and usually scales off as the aecia develop. During aecia

development, several layers of the host cells underlying them become completely dissociated from each other as a result of the abundant hyphae between them (True 1938; Crane et al. 1995). As rust sporulation ends, a new periderm forms in the gall tissue, with periderm formation being completed by fall (True 1938). The layer of aecia-bearing tissue in young galls is in turn usually exfoliated before winter or prior to the following sporulation season (True 1938; Moltzan and Blenis 1999).

Abundant rust mycelium occurs in all living host tissues near the center of a gall, whereas no rust mycelium is found more than 1cm from a gall (Peterson 1960). Hyphae may extend along the wood rays inward through many annual rings whereas relatively few hyphae leave the rays in traversing the xylem (Peterson 1960, 1961). The tangential growth of the rust often does not overtake that of the host and the gall may be limited to one side of the trunk (Peterson 1960, 1961).

Disease effect on infected trees

Tissues of the rust-infected host stem show a tendency towards hyperplasia and hypertrophy, which result in a woody swelling of the stem. In the infected cortex, the host cells, particularly the cells invaded by haustoria, are more spherical and regular in outline compared to those in normal cortex (True 1938). The annual rings in the gall are thicker than those of the uninfected stem portion. This mainly results from an increase in the proportion and number of parenchymatous cells in both phloem and xylem

elements (True 1938). Furthermore, the tracheids in the gall xylem are often deformed and shortened. Pitting of the tracheids is more frequent in gall tissue than in normal wood. The radial rows of xylem and phloem lose something of the orderly appearance seen in cross sections. There are suggestions that hyperplasia is mainly due to stimulation of the lateral meristem by the rust infection (Peterson 1961, 1967), although the actual mechanisms by which the rust induces the cambium malfunction of the rust-infected pine stem are not clear. The ultra-structural modification in the rust-haustorium-invaded cells (Hopkin and Reid 1988) may indicate the abnormal behavior of the cells results from the rust haustorium invasion.

The gall tissues contain greater amounts of nitrogen, sulphur and phosphorus, and lesser amount of silicon, than rust free wood. There is an increase in the number of vertical resin ducts, thus making the gall wood resinous (True 1938). After a few years, most wood formed by rust-invaded cambium quickly becomes brownish dark as the starches and oils in the parenchymatous cells are replaced by tannins and the invaded trachery xylem elements cease to conduct. The discolored tissue usually occurs in the core of mature galls extending outward from the center of the gall, although the gall wood tissue most recently derived from the cambium is not affected (True 1938).

Branch galls may result in the eventual death of the distal part of the branch, although they usually cause little effect on tree growth (Gross 1983). Main stem galls can kill individual trees, reduce growth rate, affect tree form,

and result in resinous lumber (Ziller 1974). While main stem galls of seedlings usually kill the plant within a few years, many stem galls of old trees live for many years without killing the tree (Peterson 1961, Hiratsuka et al. 1988). This is especially true for galls that encircle only a small portion of the tree stem (Wolken et al. 2006).

Temporal and spatial variation of infection

One of the most striking features of western gall rust epidemiology is the extreme year-to-year variation in incidence. "Wave years", single years which account for most of the infections in a decade (Peterson 1971), presumably correspond to rare conditions of high inoculum and favorable environmental conditions at a time when the host is very susceptible to infection. Examination of the relative gall locations on stems following a wave year indicated that most galls were at a similar position along the internode, suggesting the wave year may have resulted from a single massive infection event within few days (van der Kamp 1988). As high western gall rust infection levels were found to be associated with rainy, wet seasons short periods of highly favorable conditions for infection are more likely to occur in rainy, wet seasons than in relatively try seasons (Walla and Stack 1982). Although environmental factors likely play a major role in the wave year phenomenon, inoculum availability and susceptible tissue are required for infections to occur. Thus factors such as age-related resistance and gall

inactivation may limit the occurrence of wave years of infection by substantially reducing susceptible tissue and inoculum level.

The frequency of gall rust infection on lodgepole pine decreases rapidly with height above ground and becomes rare after trees reach 8 meter (van der Kamp 1988; Blenis and Duncan 1997). The number of galls per meter stem length decreased from 1.4 at 1 and 2 meter to 0 at 7 and 8 meter above the ground (van der Kamp 1988; van der Kamp et al. 1995). Four adjacent pairs of younger (average height of the examined internode = 2.1 meter) and older (average examined internode height = 6.2 meter) stands were sampled. The numbers of stem galls /10 meter of shoot length in the four pairs of older and younger stands were 1.6 and 4.1; 2.8 and 3.5; 6.5 and 10.3; and 1.3 and 2.5, respectively. On average, 67% more galls were present on the examined internodes in the younger stands (Blenis and Duncan 1997). Since these observations would not have been confounded by the wave year effect, the decline in infection with tree age was viewed as a general phenomenon (Blenis and Duncan 1997). At least three mechanisms are possibly responsible for this phenomenon: a decrease in susceptibility, a decrease in inoculum, or the occurrence of a less favorable microclimate for infection. However, none of these possible constraining mechanisms have been tested for the western gall rust – lodgepole pine pathosystem.

Constraints on infection

Seasonal shoot development constraint

The developmental stage of lodgepole pine shoots influences shoot susceptibility (Kojwang and van der Kamp 1992; Moltzan et al. 2001a). Infection levels following artificial inoculation were higher among shoots having needles just emerging from needle shelths than those shoots that were 2 weeks older (Kojwang and van der Kamp 1992). Furthermore, inoculations of a series of shoots of different elongation stages showed that the estimated susceptibility of shoots increased from 94% of maximum at 45% shoot elongation to maximum susceptibility at 90% shoot elongation, indicating shoots were most susceptible within these elongation stages. Beyond that range of elongation stages, the shoots were likely protected from infection by bud scales at earlier elongation stages and by periderm formation at later elongation stages (Moltzan et al. 2001a). The period of maximum sporulation was approximately coincidental with the period of maximum susceptibility; thus the risk of infection in the field would be highest at the stage when the tree shoots are about 50% to 90% elongated.

Effect of tree maturation on infection

For the gall rust - Monterey pine (*P. radiata* D. Don) pathosystem, shoot susceptibility was different between 1) seedlings, 2) rooted cuttings from periodic hedging to heights of 0.5, 1, 2, and 4 m of donor plants and 3) from rooted cuttings taken from free-growing trees at heights of 6 and 8 m. The seedlings had heavy infection, the cuttings from free-growing trees had no infection, and the hedged cuttings had intermediate levels of infection, with

decreasing infection with increasing hedge height (Power et al. 1994). Over twice as many galls were formed on the stecklings generated from donor monterey pine by hedging at a 0.5 m height than on the stecklings obtained from 2 m tall trees of the same clone (Zagory and Libby 1985). Since hedging maintains shoot juvenility, this increase in infection was viewed as reflecting the decrease in resistance associated with the change in maturity occurring over a height differential of 1.5 m. However, similar important information is lacking for the western gall rust-lodgepole pine pathosystem.

Genetic resistance

The susceptibility of lodgepole pine to western gall rust varies significantly among families within stands. Studies of provenance-test plantations exposed to local, natural rust inoculum sources, suggested that there were significant differences in resistance among families (Martinsson 1980; Yanchuk et al. 1988; Hoff and Sun 1994; Wu et al. 1996). By inoculating grafted clones of large trees, significant family effects on susceptibility were also evident (Kojwang and van der Kamp 1991). Using greenhouse inoculation of seedlings, which were presumably at the most susceptible stage of their life, a significant family effect was found for seed sources from near Hinton, Alberta (Blenis and Pinnel 1988; Blenis et al. 1993) and from near Prince George, B.C. (Kojwang 1994). The view that such differences in resistance play a role in limiting rust incidence in the field was supported by the fact that the degree of resistance among progeny was

consistent with differences in infection among their parent trees in the field (Blenis and Pinnel 1988). Nonetheless, differences in infection frequency between the progenies of heavily infected and lightly infected trees from the same site were quite small, suggesting that the intensity of selection might be low. On the other hand, the gall rust-lodgepole pine pathosystem appears to have other constraints such as mycoparasites. In this mycoparasite -involved pathosystem, simulation modeling showed that if rust incidence is regulated by the abundance of the mycoparasite, the effect of host resistance in reducing infection is less pronounced than if the pathosystem is not regulated by mycoparasites. Therefore, disease severity may be jointly controlled by mycoparasites and host resistance (van der Kamp and Blenis 1996).

Secondary fungal constraints

The fungi that inhabit rust galls commonly cause gall inactivation. Three fungal pathogens were recorded on the rust galls of monterey pine (Byler et al. 1972b). These fungi caused discoloration of the gall tissue and both rust and gall tissues were killed. The lesions caused by *Gibberella lateritium* (Nees.) Snyder and Hansen often became inactive before the gall cambium was killed and were subsequently incorporated into the dead gall bark. *Diplodia pinea* (Desm.) Kicks had intermediate pathogenicity. It frequently killed galls, but at other times the fungus became inactive after killing only a portion of the gall. *Nectria fuckeliana* Booth appeared to be a virulent gall pathogen. It invaded and killed the entire gall. The gall mortality that was associated with the

parasitic activities of these fungi was often widespread and thus may have significantly reduced the inoculum potential in western gall rust - Monterey pine pathosystem (Byler et al. 1972a). *N. fuckeliana* was present on most of the dead and damaged galls in some locations; therefore, it might play an important role in controlling rust populations locally (Byler et al. 1972a, b).

Scytalidium uredinicola Kuhlman, Carmichael, & Miller is a common and destructive mycoparasite of western gall rust of lodgepole pine in western Canada (Hiratsuka et al. 1979; Tsuneda et al. 1980; Moltzan and Blenis 1999). *S. uredinicola* greatly reduced the viability of gall rust spores and impeded rust spore release with its mycelium (Moltzan et al. 2001b). It appears early in the spring when rust spores are first released and pines are at their peak of susceptibility (Moltzan et al. 2001a, b); thus it is likely an important biotic constraint on gall rust inoculum production. Although it has been considered an important potential biological control agent for western gall rust (Hiratsuka 1991; Currie et al. 1995; Moltzan et al. 2001b), its ability to perennially colonize rust galls has not been well studied.

Disease control and management

Disease control in plantations

Plantations have increasingly become common in lodgepole pine management. Growing disease-free and resistant stocks reduces disease spread from nurseries and protects valuable regeneration from on-site disease damage.

Nurseries may be placed where there is no natural infection among nearby trees or, alternatively, infected trees in and around nurseries may be completely removed (Hiratsuka and Powell 1976). Susceptible nursery stocks that are exposed to heavy spore loads may be protected with fungicide sprays during the sporulation period. Dithane M-45 and Triton are effective in protecting *P. sylvestris* from infection (Kistler and Merrill 1978; Wenner and Merrill 1988). For a single application, best control occurs when the fungicide is applied as needles begin to emerge from fascicle sheaths. To protect susceptible trees for a whole infection season, multiple sprayings should be applied to achieve thorough coverage of the new growth until the needles have reached about 75% of maximum length, which is about when spore dissemination ends (Merrill and Kistler 1976). For tree farms where high-value ornamental pines are grown, similar disease control measures such as avoidance or elimination of natural inocula, and fungicide sprays may be applicable (Hiratsuka and Powell 1976).

Selection and breeding for genetically-resistant stocks may be a long term option for disease control in plantations. However, the identification of genetically-resistant trees is often challenging. A disease-free tree in the field might not necessarily be a resistant tree if the tree has merely escaped infection. Such a probability could be quite high if the disease level of surrounding trees is low and there is high variability in infection because of other factors such as nonrandom distribution of inoculum and spatial variation in microclimatic factors that influence spore dispersal and infection (van der

Kamp 1990). Although DNA markers of major resistance genes may be identified and be used for identifying resistant trees (Li and Yeh 2002), the procedure may be complex and time-consuming. Artificial inoculation methods that maintain favorable infection conditions may test the true resistance of lodgepole pine seedlings within short periods (Blenis and Pinnell 1988; Myrholm and Hiratsuka 1993), although inoculation methods for testing lodgepole pine trees against western gall rust in field conditions have not yet occurred.

Disease control in natural stands

Natural regeneration of *P. contorta var. latifolia* often results in over-stocked, dense stands (Johnstone and Cole 1988). Although precommercial thinning provides a silvicultural opportunity to improve stand yield, the selection of potential crop trees in western gall rust-infected stands will need to take the disease impact into account to secure an acceptable stand during post-thinning stand development.

As post-thinning infections decrease with increase of thinning age, delaying precommercial thinning is recommended to reduce the impact of western gall rust in the rust-infected stands (Blenis and Duncan 1997). Preferential removal of infected trees during precommercial thinning should further reduce the inoculum availability for the remaining trees. As larger, faster growing trees are often prone to infection, special effort should be made to identify infected trees for removal (Bella 1985; Navratil and Bella 1988). However, removal of all infected trees during thinning may be difficult as a

high incidence of small galls (likely not easily seen) may occur in the stands to be thinned. Because inoculum potential could be high after thinning and because post-thinning infections will directly damage the potential crop trees, it is important to know both the susceptibility of the remaining trees and the potential of remaining galls to produce spores after thinning in order to estimate the risk of post-thinning infections.

Research objectives

There were two main objectives of this study:

1. Examine the relationship between the frequency of main stem infections of lodgepole pine and tree age, and explore the anatomical features that may be responsible for such changes in infection frequency.

2. Locate *S. uredinicola* within galls in relation to the periderm, and examine the effect of this mycoparasite on periderm development and internal gall tissues.

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II. Age effect on infection of lodgepole pine by western gall rust [1]

Introduction

Western gall rust, caused by *Endocronartium harknessii* (J. P. Moore) Y. Hiratsuka, is one of the most common diseases of hard pines in western Canada (Ziller 1974). It is a pine-to-pine stem rust, that is, spores produced on pine reinfect pine shoots without the need for an alternate host (True 1938). There are few practical, direct methods for controlling western gall rust. Modified silvicultural practices, such as delayed precommercial thinning, may reduce losses (van der Kamp and Spence 1987; Blenis and Duncan 1997). This strategy is based on the assumptions that (i) stem galls, but not branch galls, can be lethal (Gross 1983), (ii) lethal stem galls arise from direct penetration of the expanding main stem leader, and (iii) the likelihood of main stem leader infection decreases as trees age.

Experimental approaches used in other tree-rust pathosystems and observational approaches in the western gall rust-lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.) pathosystem indicate that it is plausible that leader infection declines with tree age. For example, percentage infection by white pine blister rust (*Cronartium ribicola* Fischer) of eastern white pine (*P. strobus* L.) grafts aged 4, 10, 20, 40, and 80 years was 81%, 76%, 50%, 42%, and 27%, respectively (Patton 1961). Over twice as many

[1] This section has been published. Peter Blenis and Wuhan Li. 2005. Incidence of main stem infections of lodgepole pine by western gall rust decreases with tree age. Can. J. For. Res. 35: 1314-1318.

galls were formed on the stecklings from donor Monterey pine (*P. radiata* D. Don.) maintained at a 0.5 m height than from stecklings obtained from 2 m tall trees (Zagory and Libby 1985). Since hedging maintains shoot juvenility, this increase in infection was viewed as reflecting the decrease in resistance associated with the change in maturity occurring over a height differential of 1.5 m. Observational studies of the western gall rust – lodgepole pine pathosystem showed that gall frequency declined with tree height and age (van der Kamp 1988; van der Kamp et al. 1995; Blenis and Duncan 1997). In contrast with experimental studies, observational studies tend to be somewhat confounded by extreme year-to-year variation in infection frequency; there typically are infrequent "wave years" of heavy infection interspersed among several years of low infection (Peterson 1971). Observational studies also are typically unable to separate the effects of tree age from those of tree height. Age effects may result from an inherent increase in resistance with time and thus might be expected to be expressed over a range of conditions. In contrast, height effects might result from differences in inoculum concentration and/or microclimate. Given the complexity of spore movement and regional variability in weather, such effects on infection might be less consistently expressed than age effects. This study presents results of an experimental study done to test the hypothesis that stem infections decrease with tree age if inoculum concentration and environmental conditions are held constant.

Materials and methods

Four lodgepole pine stands, 5 to 125 ha in size and lying within 5 km of each other, were selected near Hinton, Alberta. The stands had been harvested between 1986 and 1989, and allowed to regenerate naturally, after drag scarification. Natural regeneration was somewhat uneven, with shorter, and presumably younger, trees growing among the larger ones. My goal was to evaluate the effect of age on main stem infection. However, since age could only be determined following destructive sampling, I selected the shorter and taller trees (average heights of 62 cm and 258 cm, respectively; Table 1) from each stand in the hopes of maximizing the range of tree ages. All trees were unshaded and free from injury or disease. Inoculations were performed in one stand in 1999. In 2000, additional inoculations were performed on different trees in that stand and in the other three stands, to provide a total of five replicated inoculations.

Spores for inoculations had been collected from within 30 km of the inoculated stands and stored in liquid nitrogen, as described earlier (Moltzan et al. 2001). Germinability, which was tested before and after each inoculation by placing spores on 1.5% water agar, remained above 90% in all inoculations. The length of the terminal leader of selected trees was measured and then inoculated by using a camel's hair paint brush (Moltzan et al. 2001) to apply spores evenly over the elongating shoot. Shoots were then misted with distilled water until just before runoff, and covered with a plastic bag containing a mass of wet cotton. The inoculation bags were then tied to the shoot and aluminum foil was placed over the bags to reflect sunlight. After 41

- 45 h, bags were removed. Two years after inoculation, inoculated trees were cut and a disk was removed from the base of each tree to permit age determination (Fig. II-1). Inoculated shoots were returned to the laboratory where tree age at the time of inoculation, gall frequency and the final length of the inoculated shoot at the end of the growing season were determined. Shoot elongation at the time of inoculation, obtained by dividing shoot length at the time of inoculation by final shoot length, was used as an index of shoot development at inoculation (Moltzan et al. 2001). Two procedures were performed to confirm that differences in infection between larger and smaller trees were not the result of microclimatic differences. First, spores were placed on water agar in Petri dishes, which were placed in 5-10 inoculation bags per height class during each replicate experiment. When the bags were removed, the plates were flooded with 20% $CuSO_4$ to stop germination. Germination of 100 randomly selected spores per plate was determined in the laboratory. Secondly, temperature was monitored by placing temperature probes inside one inoculation bag per height class, during each of the replicate inoculations. The probes were connected to a CR-21 micrologger (Campbell Scientific Inc., Logan, Utah) which recorded temperature at 60- and 30- min intervals in 1999 and 2000, respectively, and provided hourly averages.

Two statistical analyses were performed. Logistic regression was used to determine the relationship between tree age and the likelihood of infection, and multiple regression was used to determine the relationship between tree age and the number of galls per 10 cm of shoot length at the time of

inoculation. For logistic regression, the relationship between age and infection was statistically adjusted for replicate, shoot length, and elongation by including these variables in the model. The latter term was included, since infection frequency changes with percent shoot elongation (Moltzan et al. 2001). The quadratic effect of age (Age^2) was tentatively added to the model to detect nonlinearity in the relationship between age and infection above and beyond that already captured by the inherent nonlinearity of the logistic function. However, the effect of Age^2 was not significant ($P = 0.889$) and did not improve the model goodness of fit, and thus was not included in the final model. For multiple regression, data were averaged over trees for each combination of age and replicate inoculation prior to analysis, and the effects of age and age^2 on infection frequency were evaluated following adjustment for replicate and shoot elongation. Shoot length was not included as a covariate, since its effect was already captured by the dependent variable that expressed infection as incidence per 10 cm of shoot length. For both logistic and multiple regression, the final model was selected on the basis of Akaike's information criterion (Burnham and Anderson 2002).

Although height was measured, it was not used in the analysis. Because the partial correlation between height and age, after adjusting for replicate inoculations, was strong ($r^2 = 0.82$, $P < 0.0001$), including both age and height in the analysis would not be appropriate. It was thought that the effect of height on infection frequency would be indirect and mediated through differences in inoculum concentration, microclimate, or phenology. By

experimentally controlling those factors and/or measuring them and using them as covariates I hoped to demonstrate that changes in age would be sufficient to account for changes in infection frequency with height.

To determine if anatomical features of the shoot might explain differences in infection between older and younger trees, main stem shoots of shorter and taller trees were sampled from three inoculation stands. The shoots were collected during the period of inoculation and fixed in FAA (formalin-acetic-acid) within 30 min. Tree heights were determined in the same way as for inoculated trees. Tissue blocks were taken from the basal 10% of these shoots and embedded in paraffin (Jensen 1962). The sections were stained with Safranin-Fast green and examined with a light microscope equipped with a micrometer. Periderm formation was recorded and the total thickness of the cuticle and the outer epidermal cell wall were determined by systematically measuring ten cells for each tissue piece.

Results and discussion

There was a substantial decrease in infection frequency as tree age increased from 2 to 10 years (Table II-2; Fig. II-2). Over that period, the observed number of galls per 10 cm and the predicted proportion of infected trees decreased by 85% and 88% respectively (Fig. II-2). The rate of decrease was nonlinear, becoming less as the trees aged. The quadratic term for age and the sigmoidal shape of the logistic function captured the nonlinear effect

of age on the number of galls per 10 cm and the predicted proportion of infected trees, respectively.

The negative coefficient for shoot length at the time of inoculation in the logistic model (Table II-2) indicated that infection frequency decreased with increasing shoot length at the time of inoculation. Examination of partial plots (not shown) of the relationship between gall frequency and shoot length failed to reveal the cause of this counterintuitive phenomenon. An implication of this result is that since older trees tended to have longer shoots, the use of galls divided by shoot length as the dependent variable in regression analysis might have underestimated the susceptibility of older trees. However, regression of the number of galls per tree (ignoring leader length) against age showed a significant (P=0.0012) decrease in susceptibility among older trees, with the number of galls per tree decreasing by 0.34 for each additional year of tree age. Because gall frequency decreased with increasing age, irrespective of whether or not gall occurrence was adjusted for leader length, the relationship between increasing age and decreasing susceptibility appears robust.

Previous observational studies on western gall rust also showed a decrease in main stem infections with tree age (van der Kamp 1988; Blenis and Duncan 1997), but those studies are not completely comparable to the work described here, because their focus was largely on trees older than 10 years. Because this study did not examine such older trees, it is not possible to describe a threshold age beyond which there is little chance of sustaining a

devastating wave year of infection. These results do, however, show a rapid change in susceptibility over the first decade of a tree's life, and indicate the undesirability of very early precommercial thinning that would leave inadequate stocking to compensate for the risk of high infection levels.

Temperatures in the inoculation bags did not vary greatly with inoculum bag heights (Fig. II-3) but tended to be more moderate at the greater heights, which had lower maximum temperatures and higher minimum temperatures. For both height classes, average percent spore germination was the same (92%) as was the standard deviation among Petri dishes for a given combination of replicate inoculation and tree age (3%). Thus, it is unlikely that differences in microclimate were responsible for the lower infection frequency among older, taller trees.

Shoot elongation at the time of inoculation, as a percentage of final shoot length, decreased significantly with age at the rate of about 5% per year, such that the estimated elongation for the youngest and oldest age classes were 67% and 39%, respectively. This raises the possibility that the difference in infection between older and younger trees could be the result of phenological differences: younger trees had more galls than older trees, simply because they were in a more susceptible state at the time of inoculation. Phenology, however, is an unlikely explanation for the difference in infection among different-aged trees. First, the results were statistically adjusted to account for the effect of elongation on susceptibility by including percentage of elongation as a covariate in the analysis. Secondly, shoot susceptibility is

relatively independent of percentage of elongation until the shoots are about 90% elongated; the predicted increase in infection between 39% and 67% would be only about 3% (Moltzan et al. 2001).

The decline in infection frequency with age was much steeper for gall rust (Fig. II-2) than for white pine blister rust (Patton 1961). Whereas blister rust infection of grafts from 4- and 10-year-old trees were 81% and 76%, respectively, estimated gall rust infection decreased from 77% to 11%, as age increased from 2 to 10 years. In part, this difference might be the result of the ability of white pine blister rust to infect indirectly through needle stomata. In contrast, gall rust penetrates directly through the epidermis, raising the possibility that physical changes in the epidermis or periderm with age might reduce the probability of infection (True 1938; Moltzan et al. 2001).

The well-elongated surface area at the shoot base was examined for periderm; it was only formed in one of the taller trees, and in none of the shorter ones. Thus, it is unlikely that such small difference in periderm development between shorter and taller trees was responsible for the large differences in infection. Nonetheless, the shoots of taller trees appeared to have thicker outer epidermal cell walls than those of shorter trees (Fig. II-4). It has been shown that the resistance to rust is associated with the epidermal cell wall thickness (Melander and Craigie 1927). In the Scotch pine (*P. sylvestris* L.) -gall rust pathosystem, it was observed that the thickening of the epidermal cell wall often prevents germ tube penetration (True 1938).

Therefore, the chances of infection in taller, old trees may largely reduced due to an epidermis-associated resistance in lodgepole pine.

In summary, infection frequency declined steeply as trees aged from 2 to 10 years, indicating that early, aggressive, precommercial thinning is undesirable. Since inoculum concentration, microclimatic effects, and shoot elongation were either controlled or measured and evaluated, the results of this study indicate that changes in tree age are sufficient to explain the observed tendency for main stem infection frequency to decrease with tree height.

Table II-1. Average ($\bar{x}$) and range of heights and number (n) of trees in the two height classes for five replicate inoculations.

	Short trees			Tall trees		
Replicate[a]	$\bar{x}$ (cm)	Range (cm)	n	$\bar{x}$ (cm)	Range (cm)	n
1	41	19 - 66	17	260	170 - 310	14
2	56	14 - 99	50	196	120 - 270	50
3	47	15 - 98	32	189	139 - 279	56
4	85	28 - 172	21	295	230 - 370	50
5	81	31 - 173	10	319	257 - 397	27
Avg/Total	62	14 - 173	130	258	120 - 397	197

[a] The first and fifth replicates were the same stand, with different trees inoculated in 1999 and 2000, respectively.

Table II-2. Coefficients for equations predicting the number of galls per 10 cm of shoot length and the log (odds of a tree being infected).

Galls / 10 cm of shoot				Log (odds of tree being infected)			
Effect	Coefficient	95% CI[a]	P value	Effect	Coefficient	95% CI[a]	P value
Replicate	-[b]	-	0.200	Replicate	-[b]	-	0.046
Age	-1.4	± 0.8	0.001	Age	-0.38	± 0.27	0.004
Age^2	0.08	± 0.07	0.020	Shoot length[c]	-0.124	± 0.10	0.013
Shoot elongation[d]	3.16	± 3.9	0.108	Shoot elongation[d]	1.2	± 2.3	0.290

[a] 95% confidence interval on the coefficient.
[b] Because it was used as a blocking factor, the coefficients for the five replicate inoculations are not shown.
[c] Shoot length in cm at the time of inoculation.
[d] Shoot length at the time of elongation as a proportion of its final shoot length. Although this factor was not significant it was included in the model because it improved the model fit.

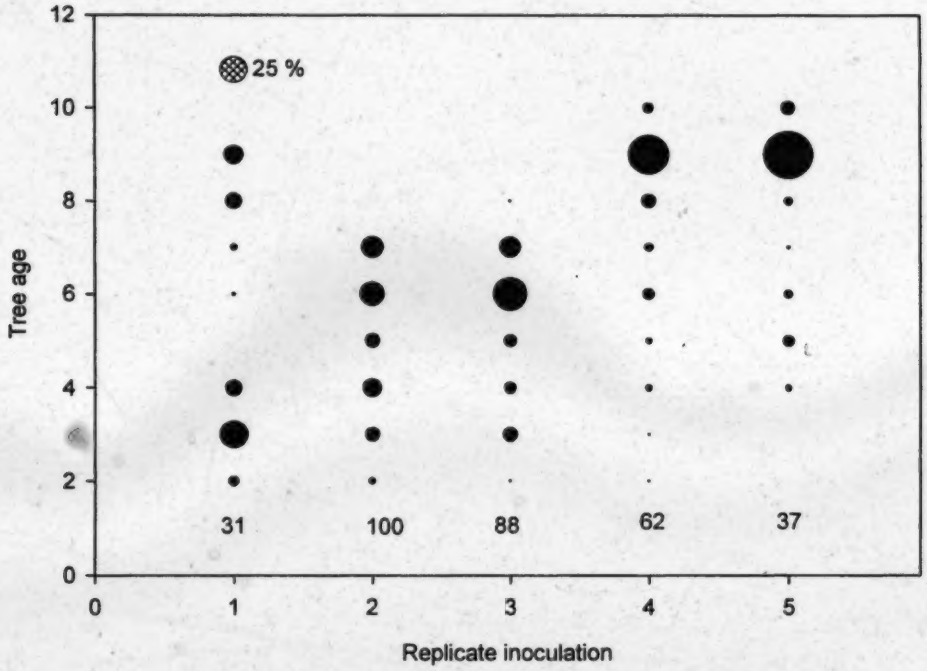

Fig. II-1. Distribution of ages of inoculated trees in the five replicate inoculations. The first and fifth replicates were the same stand, with different trees inoculated in 1999 and 2000, respectively. Symbol diameters are proportional to the percentage of trees of a particular age in any stand; the shaded bubble at the top represents 25%. Numbers at the bottom represent the total number of inoculated trees in each replicate inoculation.

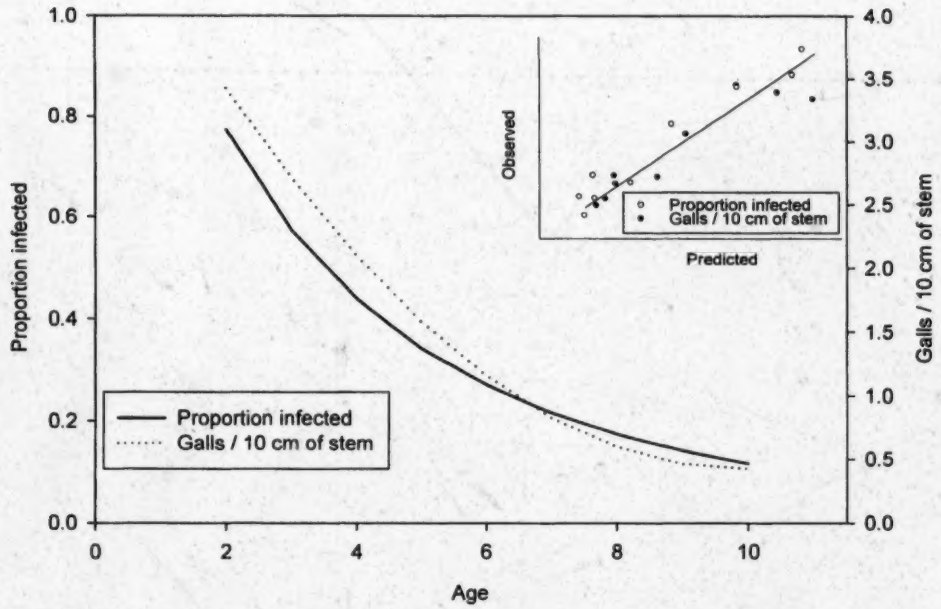

Fig. II- 2. The predicted proportion of trees infected and the predicted number of galls per 10 cm of inoculated shoot as a function of tree age, based on multiple logistic regression and multiple linear regression, respectively. Inset: observed and predictive values for the two dependent variables; the line represents equality of observed and predicted values.

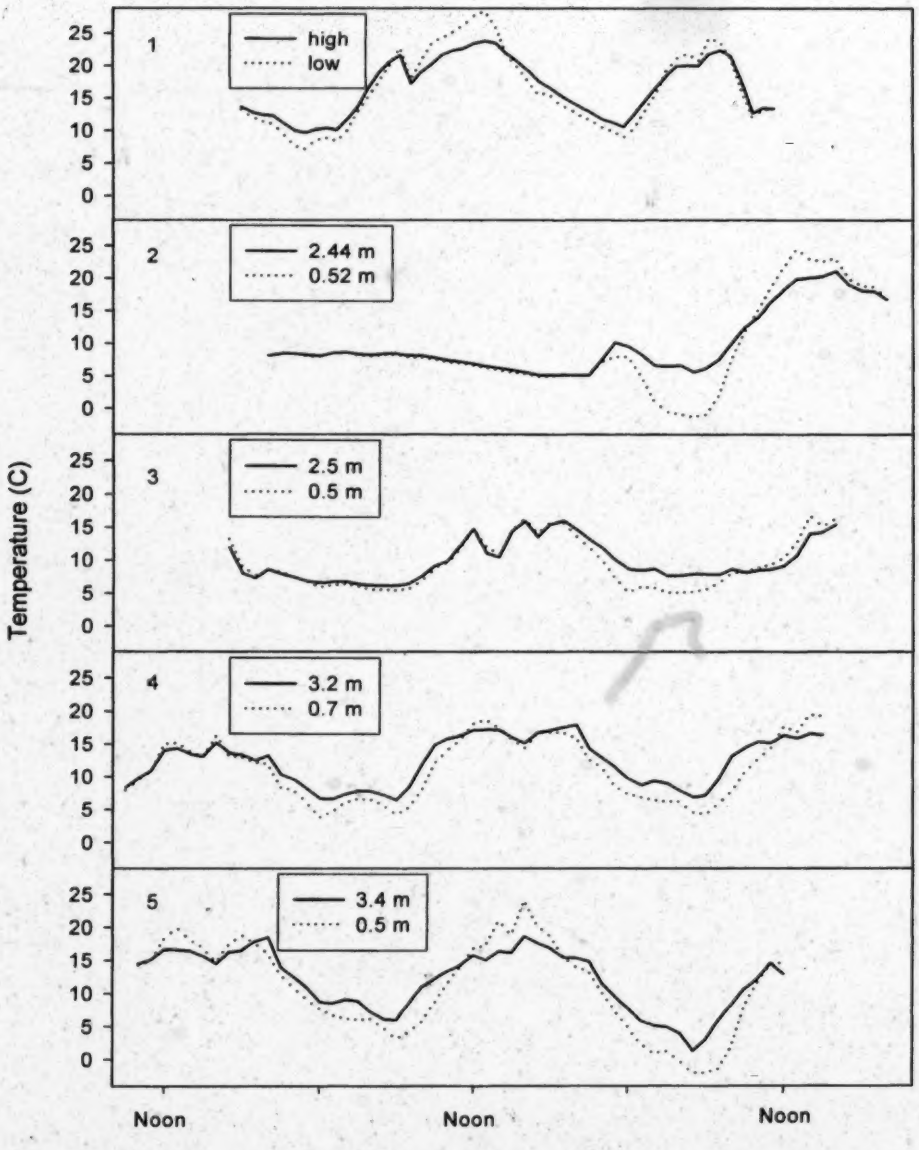

Fig. II-3. Temperatures in inoculation bags in the two height classes in the five replicate inoculations. The first and fifth replicates were the same stand, with different trees inoculated in 1999 and 2000, respectively. Heights (in metre) at which temperatures were recorded are shown for all replicate inoculations, except for the first.

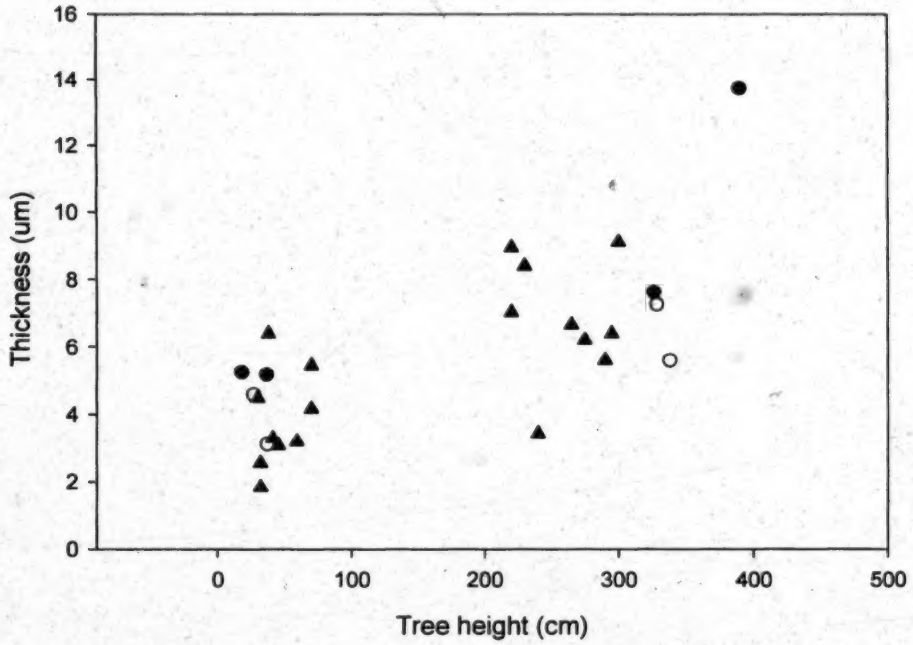

Fig. II-4. Thickness of the cutical and outer epidermal cell wall of shoots of lodgepole pine as a function of tree height. Different symbols represent four different stands.

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III. Effect of *Scytalidium uredinicola* on gall tissues of western gall rust of lodgepole pine

Introduction

Scytalidium uredinicola Kuhlman, Carmichael, & Miller is a common and destructive mycoparasite of western gall rust (*Endocronartium harknessii* (J. P. Moore) Y. Hiratsuka) (Hiratsuka et al. 1979; Tsuneda et al. 1980; Moltzan et al. 2001). The mycoparasite is an arthroconidial fungus that is vectored by a gall-inhabiting beetle *Epuraea obliquus* Hatch (Coleoptera, Nitidulidae) (Currie et al. 1995). Hyphal growth of the mycoparasite appears to be preceded by the diffusion of chemicals, which kill and disintegrate rust spores (Tsuneda et al. 1980). When a rust gall is colonized by the mycoparasite, rust sporualtion is hindered and more than 85% of the rust spores are killed (Moltzan et al. 2001). In spite of its destructive effect, the mycoparasite often does not completely inactivate the perennial rust mycelium in the cambial zone in the year of its initial infection (Tsuneda et al. 1981; Moltzan et al. 2001). Thus, the rust sporulation may re-occur if the mycoparasitic activity is not sustained in the colonized galls.

Although perennial colonization of the mycoparsite is important for understanding its impact on the gall rust, information on its ability to perennially colonize rust galls is limited. Scytalidium uredinicola colonizes the full aecial length and destroys rust hyphae beyond the bottom of the sorus (Tsuneda and Hiratsuka 1979; Tsuneda et al. 1980). The mycoparasite is able to overwinter in colonized rust galls older than 5 years and is found in the rust

sori prior to its vector-beetle presence from soil in April (Moltzan et al. 2001). Scytalidium uredinicola is recovered from both external and internal gall tissues throughout the year (Moltzan et al. 2001), although the frequency of occurrence of *S. uredinicola* in the new periderm zone of its colonized areas has not been determined. Scanning electron microscopy examination showed that *S. uredinicola* overwinters in close association with the most recently formed periderm (Moltzan et al. 2001). Therefore, its effect on periderm development and internal tissue are critical for its continuing mycoparsitic activity in the following rust sporulation season.

To better understand mycoparasitism of *S. uredinicola*, the present study analysed the occurrence of the *S. uredinicola* near the newly formed periderm, and examined the effect of *S. uredinicola* colonization on periderm development and internal gall tissues.

Materials and methods

Four lodgepole pine stands, at two sites separated by about 50 km, were selected near Hinton, Alberta. One stand was 8 years old and 4.8 ha in size. The other three were 25 ~ 30 years old and 14.9 ~ 19.3 ha in size. All stands had regenerated naturally following commercial harvesting. Generally, these four stands had a higher incidence of *S. uredinicola* than most other stands in the same area. In May and June, 1999 and 2000, stem galls with suspected mycoparasite colonization were tagged and the putatively mycoparasitized patches were further marked with a non-toxic paint on the closest position on

the stem. The marked galls were collected from May to August in 2000 and stored at -20C until used. To confirm the identity of the mycoparasite, fungal structures were removed from the surface of the colonized area and mounted in aniline blue. *S. uredinicola* was identified under a light microscope based on the morphology of its arthroconidia and hyphae (Kuhlman et al. 1976; Hiratsuka et al 1979; Moltzan et al. 2001). Those gall surface areas with abundant colonization by *S. uredinicola*, as identified through these procedures, were further marked and saved for fungal isolation as described below.

Tissue blocks were taken by inserting a 1.5-cm-diameter cork borer 1cm into the mycoparasitized gall tissue. The tissue plugs included bark and some xylem tissue and were further cut longitudinally into two equal sized portions. Only one portion from each plug was used for fungal isolation. A block of the internal living tissue, which also included a few cell layers of the dead phloem or the cork cells of the last periderm, was cut beneath the rust sorus (if the new periderm had not formed) or at the new periderm (if it was present). The tissue blocks were surface sterilized in 5% sodium hypochlorite for 10 min and then in 70% ethanol for 1min. After 3 rinses in sterile distilled water, the piece was then cut into smaller pieces which were placed on carrot agar. The plates were incubated in darkness at 17C. After 10 days of incubation, fungal mycelium recovered from the tissue block was identified using light microscopy.

In late September, 2003, *S. uredinicola*-colonized gall tissues were sampled and fixed in the field. Sampling was done in the stands that had been selected previously based on high *S. uredinicola* incidence. Tissue blocks, which included *S. uredinicola* mycelium, bark, phloem, cambium, and xylem, were taken from the *S. uredinicola*-colonized areas of galls. Tissue pieces 1mm thick were taken from the center of these tissue blocks and preserved in 2.5% glutaraldehyde (Hopkin and Reid 1988). The rest of the tissue block was kept in ice and brought to lab to confirm colonization by *S. uredinicola*. Galled tissues (without *S. uredinicola* colonization) were also sampled and fixed in the same manner. They were used as controls to identify differential responses in the *S. uredinicola*-colonized gall tissue. To prepare resin sections, the gall tissue blocks fixed in glutaraldehyde were further fixed in OsO4, dehydrated in serial concentrations of ethanol or acetone, and embedded in resin (Epon 812). Sections of 1um were made with a glass knife using an ultra-thin-section microtome. The sections were stained in toluidine blue and examined using light microscopy.

Two- and three-year-old galls were collected in early June, 2002 before rust sporulation occurred. The galls were put in a plastic bag, kept in ice and processed within 3 days. Galls were washed in 5.25% (w/v) sodium hypochlorite for 10 min, transferrd to 80% ethanol for 1 min, and then aseptically rinsed for 1 min in at least three changes of sterile distilled water (Lundquist and Walla 1994). A thin layer (< 1 mm) of the overlying bark was removed and the phloem and cortex from immediately under the excised bark

were cut into 1- to 1~2 mm^3 cubes and placed on tissue culture medium plates (Murashige and Skoog 1962) which were incubated at 18C in continuous darkness. Tissue culture growth became visible after about 2~3 weeks; the culture was transferred to fresh medium one month later. The gall tissue culture was inoculated with *S. uredinicola* mycelium, which had been cultured on Carrot Agar medium. The interface of *S. uredinicola* hyphae and gall culture cells were photographed under a dissecting microscope and then excised and fixed in FAA (formalin-acetic-acid). Paraffin sections were prepared from the fixed materials for light microscopy.

Results and discussion

The presence of *S. uredinicola* in the external and internal gall tissues has been demonstrated previously. Although *S. uredinicola* hyphae were external to the periderm, the mycoparasite was isolated from beneath the last periderm (Moltzan et. al 2001). Tissue isolation in my study showed that, in August, *S. uredinicola* was present near the periderm for 48% of the colonized areas. These results are consistent with those in the previous study (Moltzan et al. 2001), and provide further evidence that *S. uredinicola* in many of the colonized galls was established near the newly formed periderm by August (Table III-1). The occurrence frequency of the mycoparasite beneath rust sori increase from May and June before the new periderm formation (Table III-1), indicating that *S. uredinicola* was gradually moving into the gall through the growing season.

The mycoparsite-colonized gall area appeared to have thinner periderms than the *S. uredinicola*-free gall areas of a similar aged gall (Figs. III-1, III-2); thus it is likely *S. uredinicola* colonization may suppress periderm development. Nevertheless, the internal gall tissues of the colonized area appeared to be as normal as those of the non-colonized area, suggesting that the mycorparsite was compatible with the internal gall tissues. Similarly, although the *S. uredinicola* hyphae were present over the gall tissue culture for a few days and among the surface cells of the culture, the pine cells did not showed any sign of necrosis or any obvious defense response (Figs. III-3, III-4), suggesting that the pine cells were compatible with the presence of *S. uredinicola.*

It has long been known that endophytic fungi infecting grasses provide protection for their hosts by producing chemicals that inhibit herbivory (Clay 1988). *S. uredinicola* appeared to have little negative effect on the tree, other than slightly reducing periderm thickness, thus suggesting the possibility of a defensive mutualism between *S. uredinicola* and lodgepole pine, whereby the mycoparasite act as an endophyte of the rust gall to protect lodgepole pine from western gall rust.

Table III-1. Frequency of occurrence of *S. uredinicola* near the rust-sorus-formation zone in galls collected during different months.

Month	Average Gall age (Range) in years	#of galls[a]	# of tissue samples[b]	New periderm	% samples with *S. uredinicola*[c]
May	14 (9-23)	6	24	No	33
June	10 (6-18)	4	29	No	83
Aug	19 (15-21)	11	27	Yes	48

[a] Number of galls examined.

[b] Total number of locations examined over all galls.

[c] Percentage of examined locations that yielded *S. uredinicola*

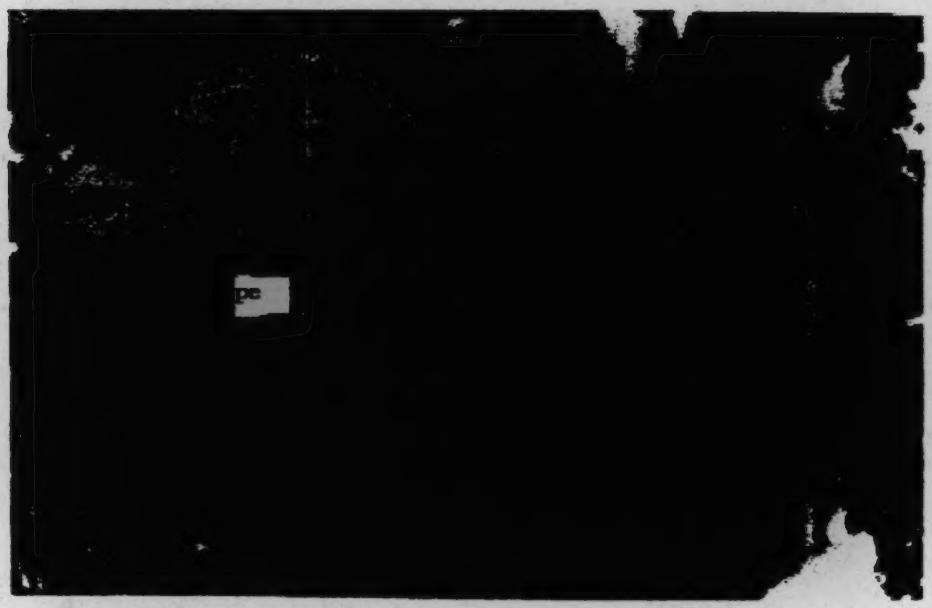

Fig. III-1: Periderm of a *Scytalidium uredinicola*-colonized gall. pe = phellem.

Fig. III-2: Periderm of a gall without *S. uredinicola* colonization. pe = phellem, pl = phloem.

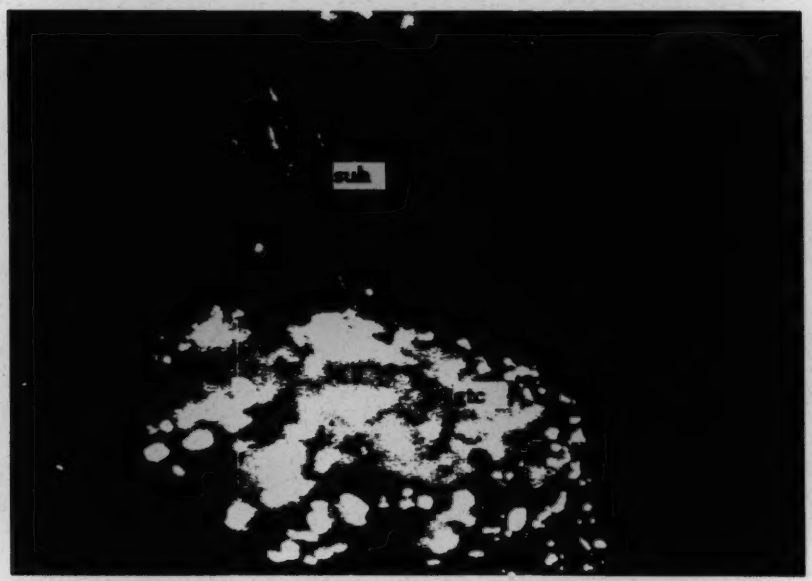

Fig. III-3. Colonization of a gall tissue culture (gtc) by *S. uredinicola* hyphae (suh).

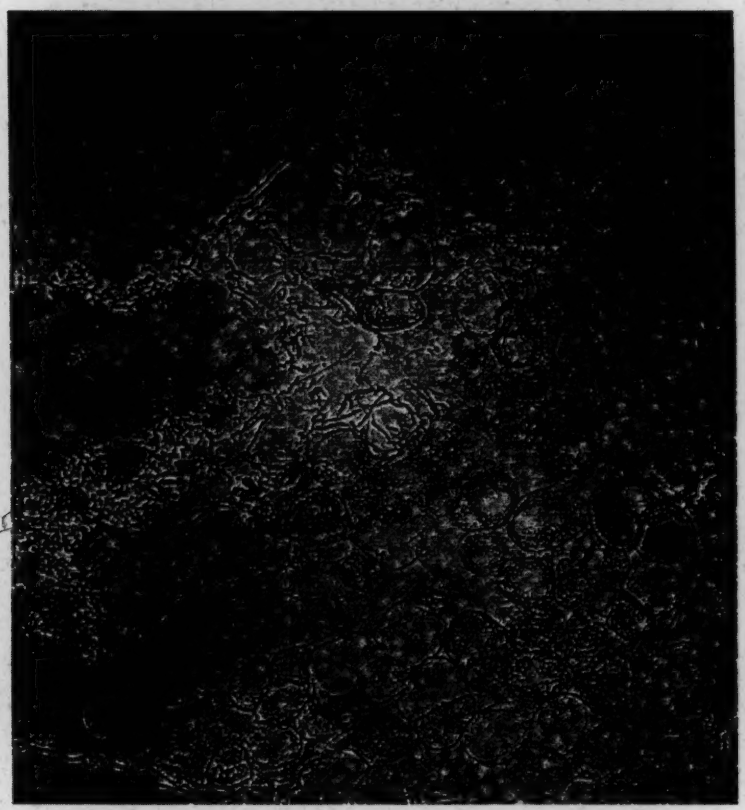

Fig. III-4. A cross section of a gall tissue culture(gtc) colonized by *S. uredinicola* hyphae (suh).

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VI. General discussion

Previous field studies have shown that the risk of infection decreases with stand age and height (van der Kamp 1988; Blenis and Duncan 1997), and this has led to recommendations to delay precommercial thinning (Blenis and Duncan 1997). If observed declines in infection frequency had occurred merely because of changes in microclimate or inoculum potential, it is possible that the reduction in infection over time might not be realized in situations in which inoculum potential and microclimate differed from those encountered during those studies. By demonstrating that decline in infection is inherently related to characteristics of trees, the phenomenon would be expected to be realized under most situations.

The decline, with tree age, in susceptibility to infection by western gall rust, was quite steep, suggesting that the amount of susceptible tissues would be expected to substantially decrease with tree growth. This tendency for susceptibility to decrease with age may be more important in natural, dense stands where natural pruning would largely eliminate branches in the lower crown, than in plantations where branches will likely survive much longer. This may be one of the reasons why western gall rust is often more prevalent in plantations than in natural stands. Furthermore, dense natural stands will likely constrain rust spore dispersal within and between stands due to tree screening effect and low air movement, whereas low-density plantations may favor western gall rust development by providing favorable environmental conditions for spore dispersal and likely for infection.

Scytalidium uredinicola is a common mycoparasite of western gall rust. It intensively suppresses rust sporulation and largely reduces rust spore viability. The mycoparasite is sustained on colonized galls from year to year likely by growing inwards beyond the rust sori during rust sporulation and by overwintering in close association with the subsequent aecia formation layer (Tsuneda et al. 1980; Moltzan et al. 2001). Furthermore, the suppression of periderm development and the rust compatibility with gall tissues suggest that the mycoparasite may have evolved an ability to persist in the colonized gall for several years, thus controlling the inoculum potential for a long time. The compatible relationship of the gall tissue and *S. uredinicola* may suggest an unique mycoparasite-lodgepole pine mutualism whereby the mycoparasite has evolved an endophytic ability in the gall tissue to protect lodgepole pine from western gall rust.

The increasing susceptibility with decreasing age of lodgepole pine suggests that the lodgepole pine stands are likely at highest risk of infection during the early stage of their establishment. Therefore, the critical factor for the rust epidemic during this stage is inoculum availability for the young stands. Sporulating galls from the nearby old stands will provide a major inoculum source, as good sanitation of the rust often results during stand replacement by wild fire or clear cut. Nonetheless, if *S. uredinicola* is common in the old stands it may critically reduce the inoculum level in the old stands, thus constraining the rust population development during this stand development stage. With further stand development, trees then become

increasingly resistant with age and the colonization frequency of *S. uredinicola* also increases with gall age (Moltzan and Blenis 1999). Therefore, both the age-related resistance and the persistent rust inoculum control by *S. uredinicola* are likely two major constraints of the gall rust epidemiology in lodgepole pine –gall rust pathosystem.

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